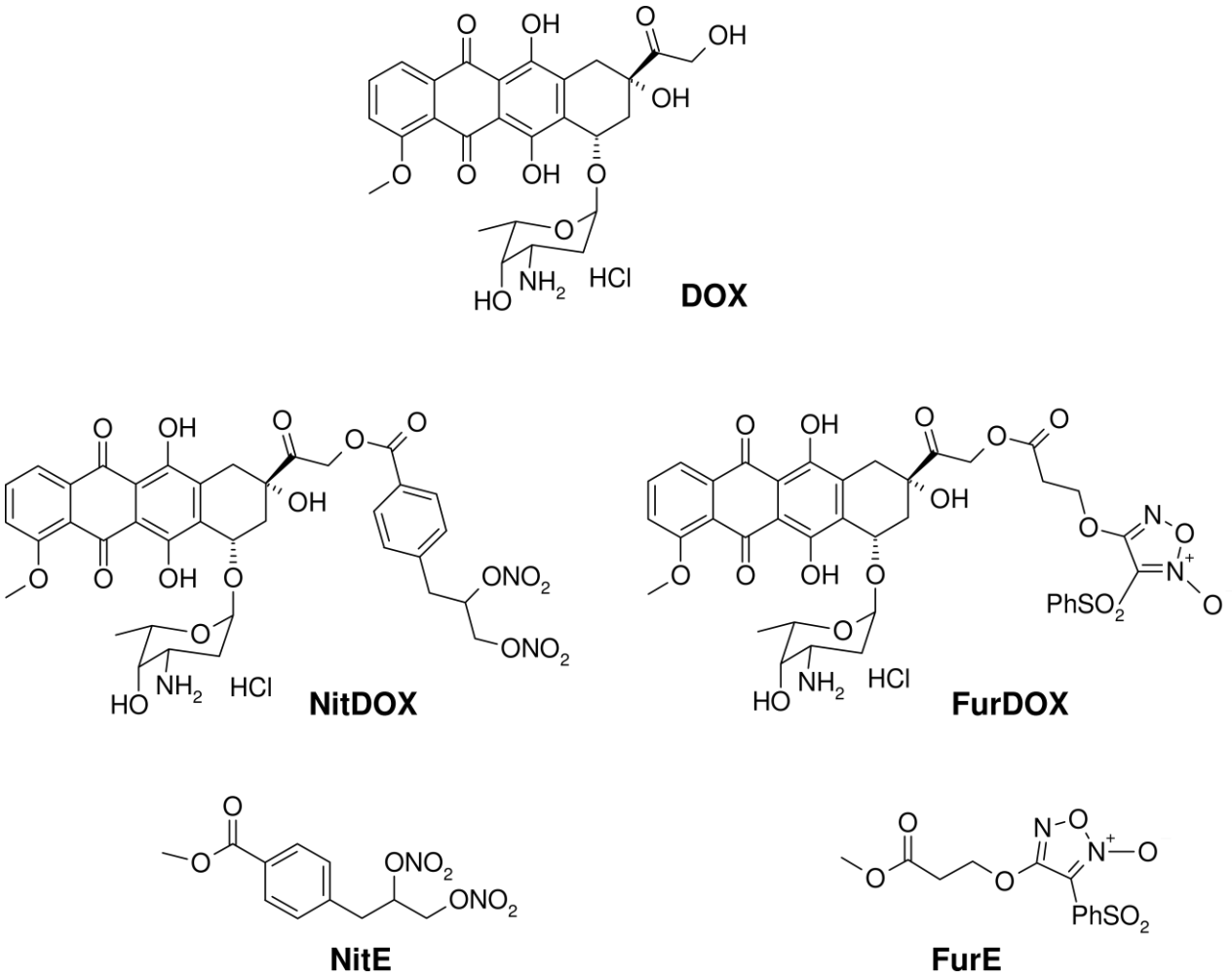
Supporting Information

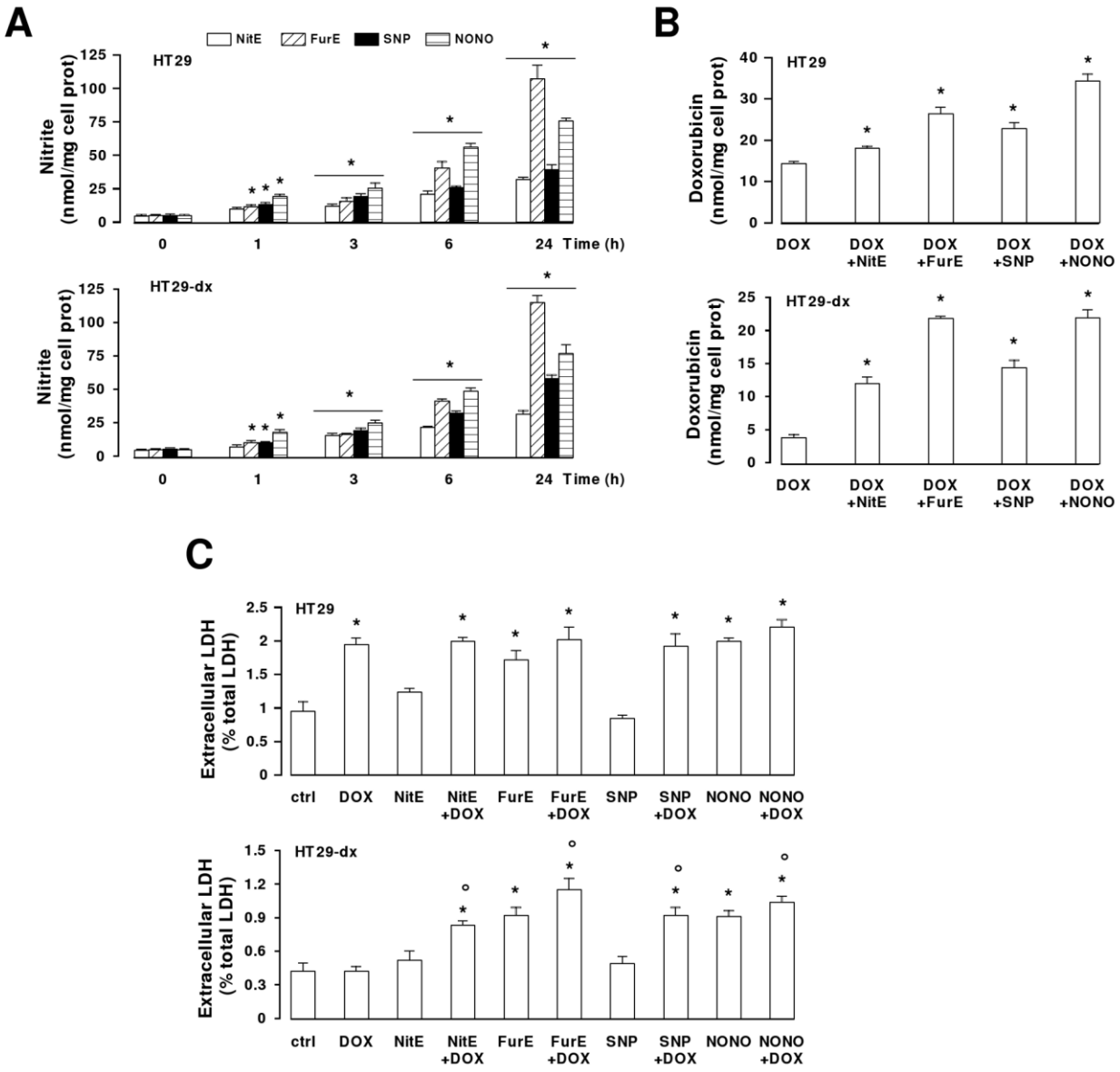
Supplementary Figure S1



Supplementary Figure S1. Structure of doxorubicin (DOX), of NO-donor derivatives nitrooxy-DOX (NitDOX), 3-phenylsulfonylfuroxan-DOX (FurDOX) and of the methyl esters of acids containing nitrooxy moiety (NitE) and phenylsulfonylfuroxan moiety (FurE).

Supporting Information

Supplementary Figure S2



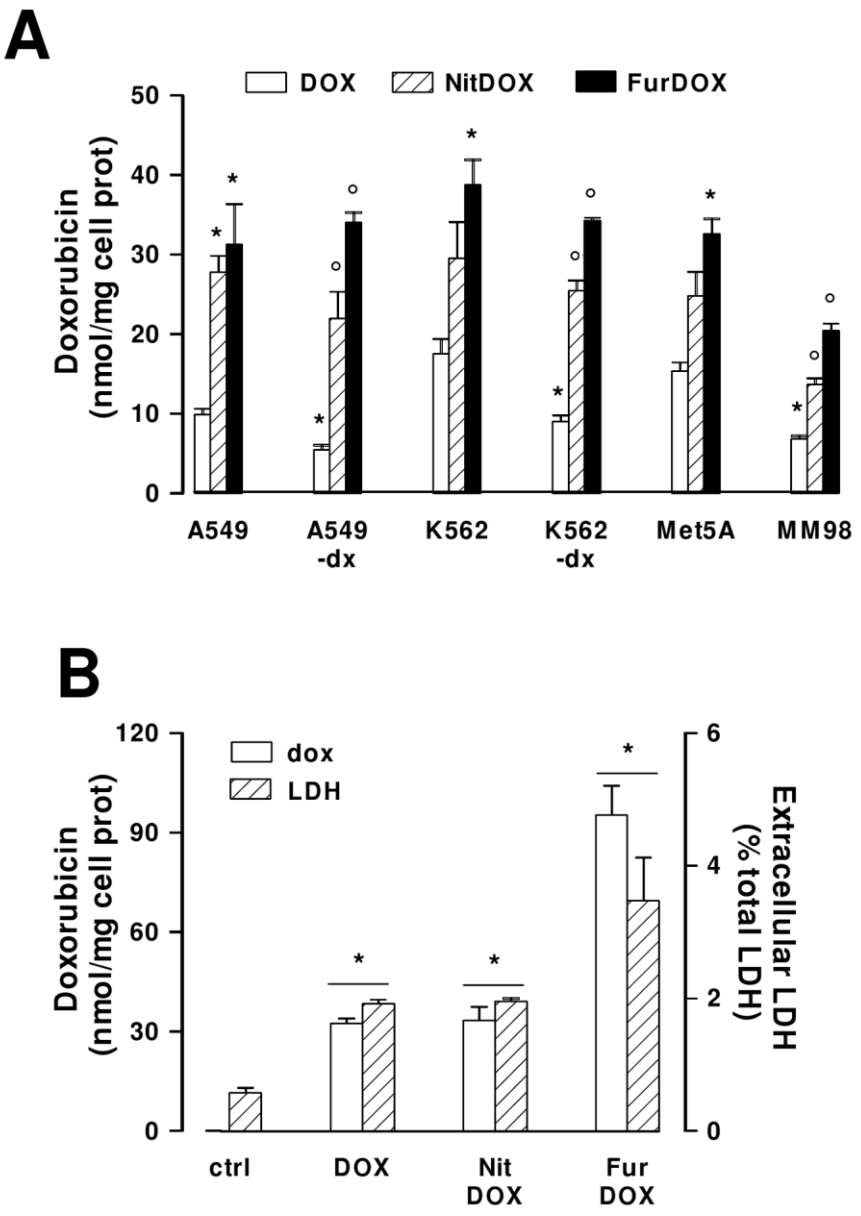
Supplementary Figure S2. Effects of the association of NO-donors and doxorubicin in drug-sensitive HT29 cells and drug-resistant HT29-dx cells.

A. HT29 and HT29-dx cells were cultured in fresh medium (*0*) or in the presence of 5 μmol/L of one of the following NO-donors: the methyl ester of acid containing nitrooxy moiety (*NitE*), the methyl ester of acid containing phenylsulfonylfuroxan moiety (*FurE*), sodium nitroprusside (*SNP*), spermine NONOate (*NONO*), for 1, 3, 6, 24 h. The amount of extracellular nitrite in culture

supernatants was evaluated spectrophotometrically. The measurements were performed in triplicate and data are presented as means ± SD (n = 3). Versus untreated (*0*) cells: * $p < 0.05$. **B.** Cells were incubated for 24 h with 5 µmol/L doxorubicin alone (*DOX*) or in the presence of 5 µmol/L of one of the NO-donors indicated in **A**. The amount of doxorubicin was measured fluorimetrically in cell lysates. Measurements were performed in triplicate and data are presented as means ± SD (n = 3). Versus *DOX* alone in the same dataset: * $p < 0.02$. **C.** Cells were incubated for 24 h with fresh medium (*ctrl*), 5 µmol/L doxorubicin alone (*DOX*), 5 µmol/L of the NO-donors indicated in **A**, alone or in different combinations. The release of LDH in the culture supernatant was detected spectrophotometrically. Measurements were performed in triplicate and data are presented as means ± SD (n = 3). Versus *ctrl* (*0*): * $p < 0.02$. Versus *DOX* alone in the same dataset: ° $p < 0.02$.

Supporting Information

Supplementary Figure S3



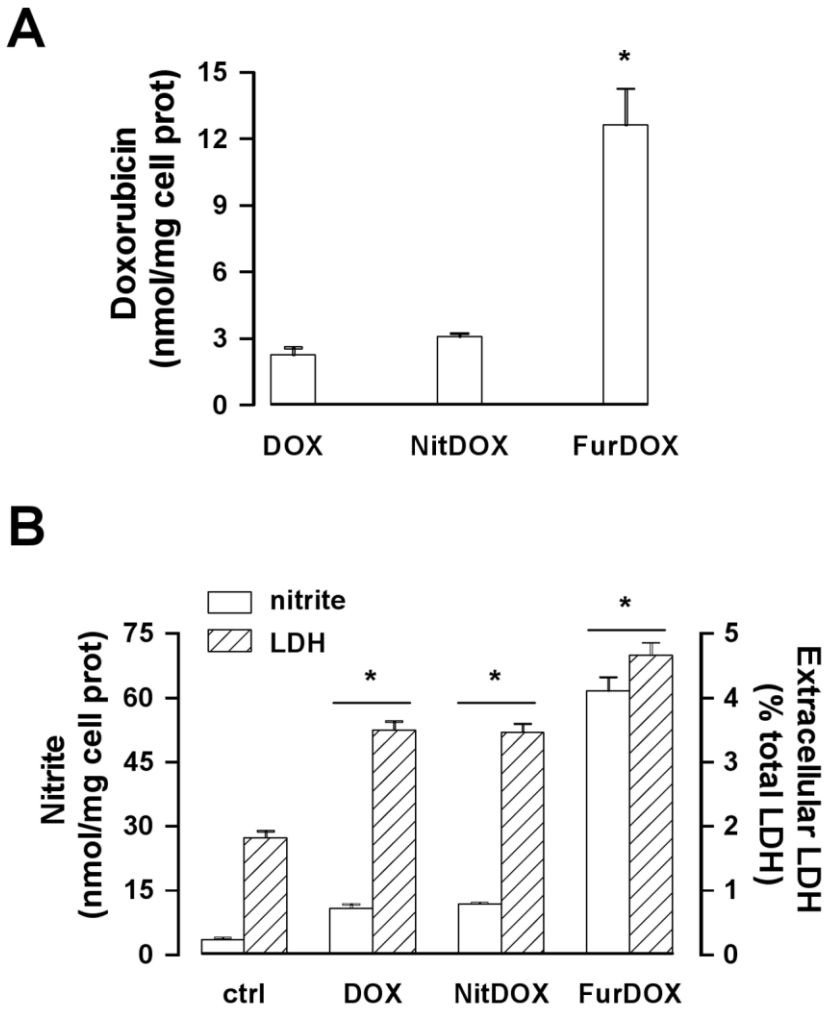
Supplementary Figure S3. Effects of doxorubicin and NO-releasing doxorubicins in drug-sensitive and drug-resistant cancer cell lines and in cardiomyocytes.

A. The intracellular DOXs accumulation was detected in chemosensitive cell lines (lung cancer A549 cells, chronic myeloid leukemia K562 cells) and in their chemoresistant counterparts (A549-dx cells, K562-dx cells), as well as in non-transformed mesothelial Met5A cells and in

constitutively chemoresistant human malignant mesothelioma MM98 cells, all incubated with 5 μmol/L DOX (*DOX*), nitrooxy-DOX (*NitDOX*) or 3-phenylsulfonylfuroxan-DOX (*FurDOX*) for 24 h. The measurements were performed in triplicate and data are presented as means ± SD (n = 3). Versus A549, K562 or Met5A cells respectively: * $p < 0.05$. Versus A549-dx, K562-dx or HMM cells respectively: ° $p < 0.005$. **B.** The intracellular accumulation of DOXs and the release of LDH were measured in rat H9c2 cardiomyocytes, grown in the absence (*ctrl*) or presence of 5 μmol/L DOX (*DOX*), nitrooxy-DOX (*NitDOX*) or 3-phenylsulfonylfuroxan-DOX (*FurDOX*) for 24 h. Measurements were performed in triplicate and data are presented as means ± SD (n = 3). Versus *ctrl*: * $p < 0.001$.

Supporting Information

Supplementary Figure S4



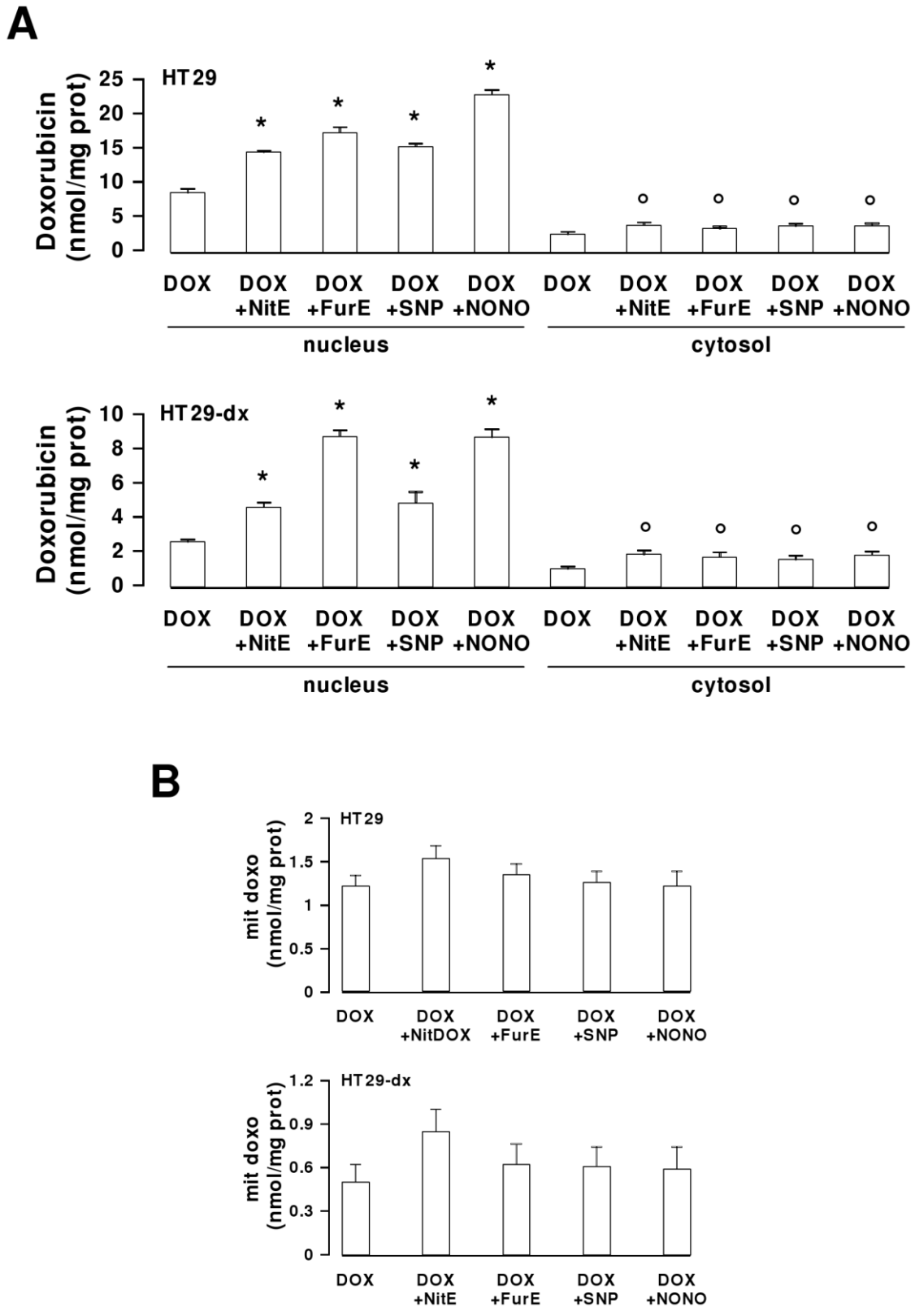
Supplemnetary Figure S4. Effects of doxorubicin and NO-releasing doxorubicins in non-transformed colon epithelial cells.

Human colon epithelial cells CCD-18Co were cultured in fresh medium (*ctrl*) or in the presence of with 5 µmol/L DOX (*DOX*), nitrooxy-DOX (*NitDOX*) or 3-phenylsulfonylfuroxan-DOX (*FurDOX*) for 24 h. **A.** The intracellular accumulation of DOX was measured fluorimetrically in cell lysates. Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 3). Versus *DOX*: * $p < 0.05$. **B.** The amount of nitrite and the release of LDH in the culture supernatants were

measured by spectrophotometric assays. Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 3). Versus *ctrl*: * $p < 0.001$.

Supporting Information

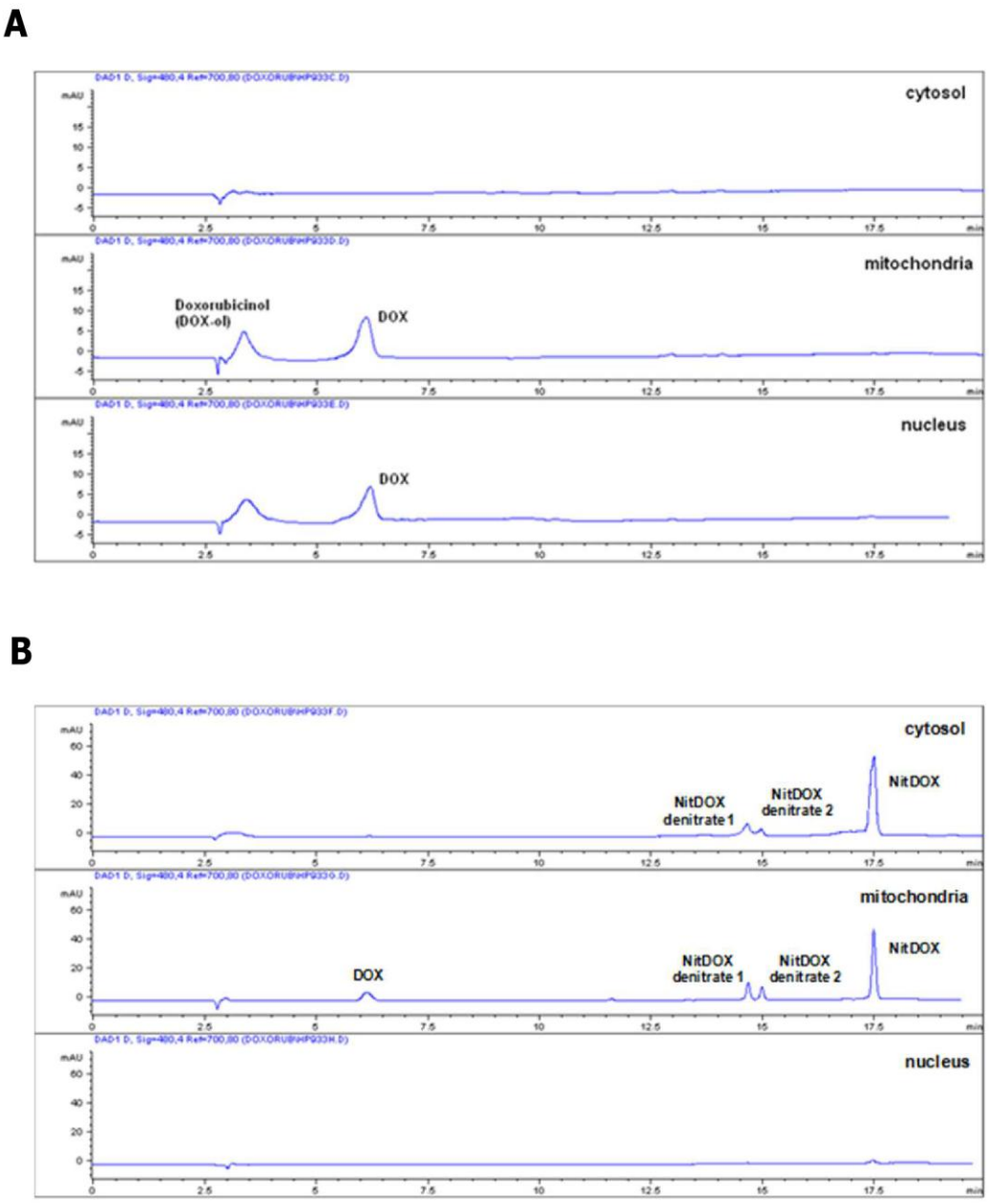
Supplementary Figure S5



Supplementary Figure S5. Effects of NO-donors on doxorubicin intracellular localization in drug-sensitive HT29 cells and drug-resistant HT29-dx cells.

A. Nuclear and cytosolic content of DOX. HT29 and HT29-dx cells were treated for 24 h with 5 μmol/L DOX (*DOX*), alone or with 5 μmol/L of one of the following NO-donors: the methyl ester of acid containing nitrooxy moiety (*NitE*), the methyl ester of acid containing phenylsulfonylfuroxan moiety (*FurE*), sodium nitroprusside (*SNP*), spermine NONOate (*NONO*). The intracellular drug content in nuclear and cytosolic extracts was evaluated by a fluorimetric assay. Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 3). *DOX* versus *DOX + NO-donors* in nucleus: * $p < 0.02$; *DOX* versus + *NO-donors* in cytosol: ° $p < 0.05$. **B.** Mitochondrial content of DOX. Cells were incubated as reported in **A**, then lysed and subjected to the isolation of mitochondria. The mitochondria extracts were analyzed fluorimetrically to measure the DOX amount. Measurements were performed in triplicate and data are presented as means $\pm$ SD (n = 3).

Supplementary Figure S6

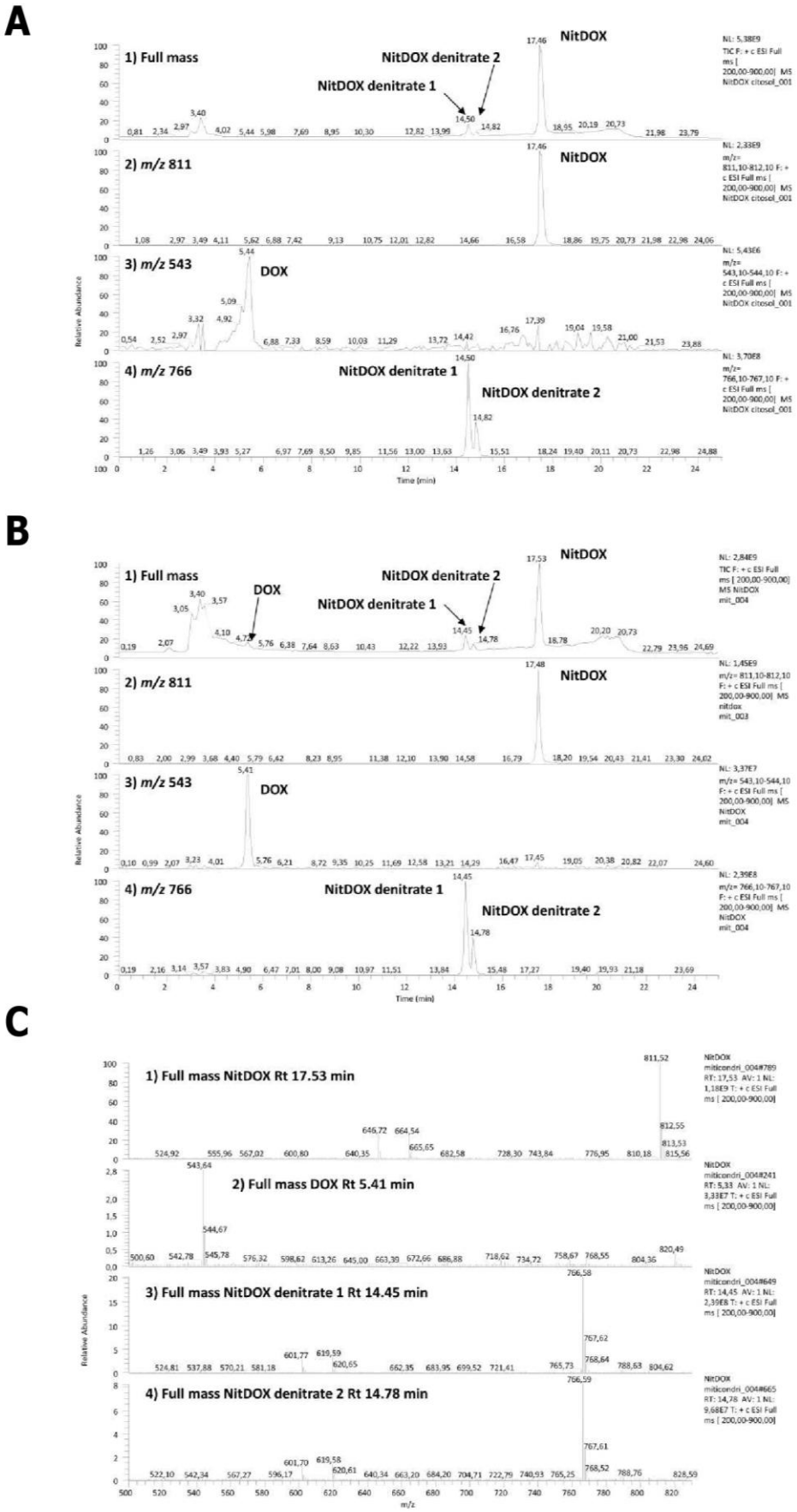


Supplementary Figure S6. HPLC analyses of cell fractions from HT29 cells incubated with doxorubicin and nitrooxy-doxorubicin.

HT29 cells were incubated for 24 h with 10 µmol/L DOX (panel **A**) or NitDOX (panel **B**), in duplicate. One series of samples was subjected to cystosol-nuclei separation, the second one was used for the isolation of mitochondria. RP-HPLC analyses was performed in mitochondrial,

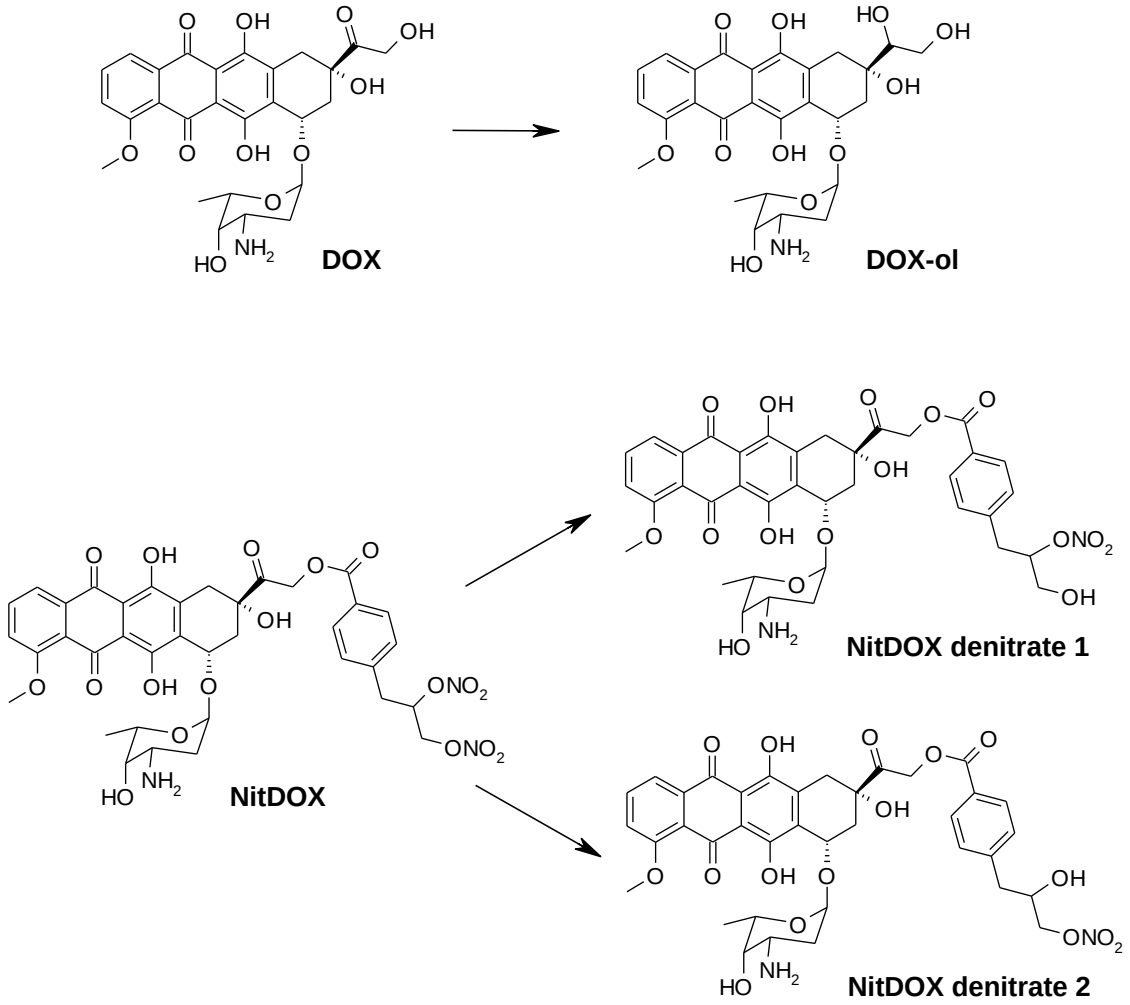
cytosolic and nuclear fractions, as described in the Experimental Section. The chromatograms reported in the figure are representative of 5 experiments with superimposable results.

Supplementary Figure S7



Supplementary Figure S7. LC-ESI-MS analyses of cell fractions from HT29 cells incubated with doxorubicin and nitrooxy-doxorubicin for 24 h with 10 µmol/L DOX: cytosolic (panel A) and mitocondrial (panel B) extracts. MS spectrum of analytes (panel C).

Supplementary Figure S8



Supplementary Figure S8. Structure of metabolites deriving from 24 h incubation of DOX or NitDOX in HT29 cells: doxorubicinol (DOX-ol) and NitDOX denitrated derivatives (NitDOX denitrate 1 and NitDOX denitrate 2).